

# Platelet secretion from dense and $\alpha$ -granules in vitro in migraine with or without aura

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## Abstract

Several studies in vivo indicate platelet activation in migraine, as reflected by increased plasma concentrations of platelet secretory products. In vitro data on platelet secretion are scant, which prompted an investigation into agonist-induced platelet aggregation and secretion in platelets from patients with migraine. Sixty two patients with migraine with aura (MA) and 41 with migraine without aura (MwA) were studied during a headache-free phase, together with 26 healthy controls. Platelet aggregation and secretion in platelet-rich plasma were induced by collagen and platelet activating factor (PAF). Serotonin was measured by high performance liquid chromatography and platelet factor 4 (PF4) with an enzyme immunoassay kit. There were no significant aberrations in platelet aggregation in those with migraine compared with healthy controls. The platelet PF4 secretion induced by PAF (1.0 and 0.1  $\mu$ M) was increased in MwA ( $p < 0.05$ ,  $p < 0.0001$ ) compared with controls, and there was a similar trend in MA (NS,  $p < 0.01$ ). By contrast, the PF4 secretion induced by collagen (0.5 and 2.0  $\mu$ g/ml) was reduced in MA ( $p < 0.01$  and  $p < 0.05$ ). Further, the MA group exhibited increased basal intraplatelet serotonin concentrations ( $p < 0.0001$ ) and increased serotonin secretion induced by both concentrations of collagen ( $p < 0.0001$ ) and PAF ( $p < 0.001$ ). The data indicate an abnormal platelet  $\alpha$ -granule secretion in those with migraine, and focus attention on PAF as a possible factor contributing to the platelet activation associated with migraine. The increased platelet content and secretion of serotonin was specific to MA, and may reflect different serotonin turnover in the two clinical migraine types.

(*J Neurol Neurosurg Psychiatry* 1994;57:557-561)

Platelet activation in association with migraine was first suggested by findings in the early 1960s. These were indicative of serotonin release from circulating platelets during the attack,<sup>1</sup> thereby initiating the still ongoing discussion on the role of platelets and serotonin in migraine. Due to the important role attributed to serotonin, platelet studies in migraine have focused; for the most part, on the serotonin-related aspects of platelet function and metabolism.

Other reflections of in vivo platelet activation in migraine are increased plasma concentrations of the platelet  $\alpha$ -granule proteins,  $\beta$ -thromboglobulin ( $\beta$ -TG) and platelet factor 4 (PF4).<sup>2,3</sup> Although these concentrations are also raised in the headache-free periods, the increases are apparently particularly pronounced in association with attacks, indicating that the postulated serotonin release from dense granules during migraine attacks is paralleled by  $\alpha$ -granule secretion.

Despite this evidence in vivo of platelet hypersecretion in migraine, there have been few in vitro studies of platelet secretion. Often, platelet aggregation has been used as the solitary measure of platelet function. Coupled measurements of agonist-induced platelet aggregation and the secretion from dense and  $\alpha$ -granules, could increase knowledge about factors causing platelet activation in migraine, and add to the understanding of the possible role of platelets in the pathophysiology of migraine attacks.

Accordingly, we determined aggregation and secretion, induced by collagen and platelet activating factor (PAF), in platelets from subjects with migraine with aura (MA) and migraine without aura (MwA) during a headache-free phase.

## Materials and methods

### STUDY SAMPLE

We studied 62 patients with MA (38 males, 24 females) and 41 patients with MwA (23 males, 18 females) (table 1). The clinical diagnosis was made from the International Headache Society classification.<sup>4</sup> None of the patients had mixed headaches or concomitant diseases. The patients with migraine were studied during a headache-free phase, and had been free of an attack for at least three days. The control sample consisted of 26 healthy volunteers (13 males, 13 females) who were matched for age and sex with the patients. Any headache within two weeks was treated with paracetamol. Apart from this, all subjects disclaimed taking any drugs (includ-

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Received 18 May 1993  
and in revised form  
9 August 1993.

Accepted 17 August 1993

Table 1 Demographic description of the study sample

	Males	Females	Age* (y)	Frequency* (attacks/y)	Duration* (hours)	Pain intensity†
Migraine with aura	38	24	27 (13-60)	5 (1-10)	12 (2-72)	1-3
Migraine without aura	23	18	33 (12-52)	36 (12-60)	14 (3-72)	2-3
Healthy controls	13	13	31 (18-57)			

\*Values are median (range).

†1 = light 2 = medium 3 = strong.

ing oral contraceptives) for at least two weeks before the study.

#### BLOOD SAMPLING

Blood was drawn from an antecubital vein at 0900 from subjects in the supine position after overnight fasting. Blood was drawn on one occasion from each male subject and, in view of the considerable variations in platelet function during the course of the menstrual cycle, on three occasions from all female subjects; in the early follicular phase, midphase (15 days after onset), and late luteal phase. The mean value of these three samples was used in the statistical evaluation.

#### PREPARATION OF PLATELET-RICH PLASMA

Blood was anticoagulated with a 1/10 volume of 3.8% sodium citrate solution (w/v) and platelet-rich plasma was obtained by centrifugation at  $165 \times g$  for 15 minutes at room temperature. The platelet number was adjusted to  $350\,000/\mu\text{l}$  with autologous platelet-poor plasma obtained by centrifugation of platelet-rich plasma at  $2000 \times g$  for 15 minutes.

#### PLATELET AGGREGATION

Platelet aggregation in 1.0 ml platelet-rich plasma was studied with a Chrono-Log aggregometer (Chrono-Log Corp, Haverton, PA, USA). Platelet aggregation was determined as the percentage change in light transmission (T) and the maximal light transmission was set with platelet poor plasma. The agonists were added in 10  $\mu\text{l}$  volumes. The aggregation velocity ( $V_{\text{max}}$ ) was determined as the maximal percentage of increase in T/min during the first minute after shape change, and maximal aggregation ( $A_{\text{max}}$ ) as percentage increase in T when aggregation had reached its final plateau. The agonists used were collagen (0.5 and 2.0  $\mu\text{g}/\text{ml}$ ) and PAF (1-O-alkyl-O-acetyl-sn-glycero-3-phosphorylcholine; 0.1 and 1.0  $\mu\text{M}$ ). PAF and collagen were purchased from Sigma Chemical Co, St Louis, MO, USA. Three minutes after addition of the agonist, the sample was rapidly transferred from the aggregation vial into an Eppendorf tube and pelleted by centrifugation for two minutes at 14 000 rpm in an Eppendorf 5415 centrifuge.

#### DETERMINATION OF PF4 RELEASE

The supernatant fraction was kept stored at  $-80^\circ\text{C}$  until analysis of PF4 by a commercial

enzyme-immunoassay kit (Asserachrom, Boehringer Mannheim).

#### DETERMINATION OF SEROTONIN RELEASE

The platelet pellet was resuspended in 1 ml of physiological saline, and the suspension was sonicated at room temperature for three minutes with a B Braun 2000 sonicator at  $-60$ -output. The suspension was then deproteinised by adding 30 mg of 5-sulpho-salicylate-dihydrate and centrifuging for two minutes at 14 000 rpm in an Eppendorf centrifuge. The supernatant was stored at  $-80^\circ\text{C}$  until high performance liquid chromatography (HPLC) mainly according to the methodology previously described in detail,<sup>5</sup> but with a modified mobile phase consisting of 125 mM  $\text{Na}_2\text{HPO}_4$ , 100 mM citric acid, 0.5 mM octyl sulphate, 0.07 mM EDTA, and 8% acetonitrile (pH 4.72). We used a Waters HPLC with Amperometric detector M-460 at +0.60 V and a reversed phase column (Supelcosil LC-18, 3  $\mu$ , 15 cm  $\times$  4.6 mm) purchased from Supelco Inc, Bellefonte, PA, USA. Two hundred  $\mu\text{l}$  of sample was added to 50  $\mu\text{l}$  of a solution containing the internal standard N-CH<sub>3</sub>-5-HT and 50  $\mu\text{l}$  of this mixture was autoinjected. The retention time for serotonin was 11 minutes and for the internal standard 15 minutes. Platelet serotonin release was calculated by subtracting the values of intraplatelet serotonin in a stimulated sample from that of the basal value in the non-stimulated sample.

#### STATISTICAL ANALYSIS

The data were analysed by mixed analysis of variance and Student's *t* test.

### Results

There were no significant differences between males and females, either in patients or controls (data not shown).

#### BASAL INTRAPATELET SEROTONIN CONCENTRATIONS

The mean basal platelet values for serotonin were significantly increased in platelets from patients with MA compared with those from controls and patients with MwA (table 2). The values in platelets from patients with MwA did not significantly differ from those of controls.

Table 2 Basal platelet concentrations of serotonin, and platelet secretion of serotonin and platelet factor 4 after stimulation with collagen and PAF in patients suffering from migraine with or without aura and in healthy controls

	Serotonin (ng/10 <sup>9</sup> platelets)			Platelet factor 4 (IU/ml)		
	Controls	Migraine with aura	Migraine without aura	Controls	Migraine with aura	Migraine without aura
Basal value	855 (450)	1397 (1168)††††****	834 (534)			
Collagen:						
0.5 $\mu\text{g}/\text{ml}$	433 (374)	703 (667)††††****	345 (255)	4196 (2300)	3460 (2094)†††*	3902 (2355)
2.0 $\mu\text{g}/\text{ml}$	543 (396)	961 (891)††††****	516 (360)	5746 (2507)	5049 (2454)††††*	5991 (3353)
PAF:						
0.1 $\mu\text{M}$	273 (391)	449 (567)†††***	235 (284)	1319 (1356)	1786 (1586)	2007 (2270)*
1.0 $\mu\text{M}$	406 (404)	654 (634)†††***	395 (324)	3170 (1875)	4346 (2664)**	5325 (3618)††††****

Values are means (SD).

\* A statistically significant difference between a migraine group and the control group.

† A statistically significant difference between the two migraine groups.

\*† p < 0.05; \*\*†† p < 0.01; \*\*\*††† p < 0.001; \*\*\*\*†††† p < 0.0001.

Table 3 The maximum velocity ( $V_{max}$ ) and amplitude ( $A_{max}$ ) of platelet aggregation induced by collagen and PAF in patients with migraine with or without aura and in healthy controls

	Controls		Migraine with aura		Migraine without aura	
	$V_{max}$	$A_{max}$	$V_{max}$	$A_{max}$	$V_{max}$	$A_{max}$
Collagen						
0.5 $\mu$ g/ml	63.9 (17.6)	59.2 (10.2)	63.0 (24.4)	58.0 (12.3)	59.8 (23.3)	56.7 (13.7)
2.0 $\mu$ g/ml	88.3 (13.3)	70.9 (10.7)	89.4 (17.6)	67.8 (4.71)	82.4 (18.4)	63.0 (15.4)
PAF						
0.1 $\mu$ M	38.6 (15.6)	15.4 (13.7)	45.4 (23.5)	20.1 (14.6)	35.0 (17.3)	16.7 (18.6)
1.0 $\mu$ M	84.5 (16.3)	56.0 (17.3)	85.4 (12.9)	58.7 (16.7)	83.4 (17.9)	51.3 (18.0)

Values are means (SD). There were no statistically significant differences between the three groups.

#### PLATELET AGGREGATION

The  $V_{max}$  and  $A_{max}$  of platelet aggregation induced by collagen and PAF did not differ significantly between platelets from any groups (table 3).

#### COLLAGEN-INDUCED PLATELET SECRETION

Collagen-induced serotonin secretion was significantly increased in platelets from patients with MA compared with both those from controls and those from patients with MwA (table 2). By contrast, PF4 secretion was significantly lower in platelets from patients with MA compared with both those from MwA and controls. There were no significant differences between platelets from patients with MwA and those of controls.

#### PAF-INDUCED PLATELET SECRETION

PAF-induced serotonin secretion was significantly increased in platelets from patients with MA both compared with those from patients with MwA and those from controls (table 2). The PF4 secretion induced by 0.1  $\mu$ M PAF was significantly increased in platelets from patients with MwA compared with controls. There was also a tendency towards an increase in platelets from patients with MA, but it did not reach statistical significance when compared with controls. The PF4-secretion induced by 1.0  $\mu$ M PAF was significantly increased in platelets from both migraine groups compared with controls. The secretion was also significantly higher in platelets from patients with MwA than MA.

#### Discussion

Our present data indicate that the characteristics of platelet function differ significantly between the two clinical types MA and MwA, as demonstrated by different profiles of stimulated secretion of platelet granules in vitro. Indeed, the substantially increased platelet content and secretion of serotonin from dense granules seems to be a specific biological marker for MA. Further, the impairment of platelet  $\alpha$ -granule secretion on collagen stimulation was found only in platelets from the MA group. On the other hand, enhancement in the PAF-induced platelet secretion from the  $\alpha$ -granules, is apparently a common feature shared by platelets from the two migraine types.

Our data thus indicate an aberrant platelet  $\alpha$ -granule secretion in platelets from patients with migraine, and the divergent platelet

responses induced by the two agonists, PAF and collagen, suggest an abnormality in transformation of the cellular signal. It may seem abstruse that the other platelet responses did not follow the same pattern, as they are generally considered to be elicited according to the potency of the stimulus in the order: aggregation, dense granule secretion, and  $\alpha$ -granule secretion.<sup>6</sup> Platelet responses do not, however, always occur in this sequential order,<sup>7-9</sup> and furthermore the proportions between dense and  $\alpha$ -granule secretion may vary at different agonist concentrations.<sup>10</sup>

Our present finding of a specific platelet  $\alpha$ -granule hypersecretion in response to PAF is in accordance with other findings in patients with migraine of an enhanced increase in platelet cytoplasmic ionised calcium ( $[Ca^{2+}]_i$ ) on stimulation with PAF, but not collagen.<sup>11</sup> Thus, the platelet hypersecretion from  $\alpha$ -granules induced by PAF may, at least in part, be connected to aberrant intracellular calcium transients and perhaps an enhancement in the coupling between the phosphatidylinositol cycle and  $Ca^{2+}$  mobilisation. This assumption is not in controversy with the diverse platelet response to collagen, as collagen only mobilises  $Ca^{2+}$  weakly.<sup>12</sup>

The mechanisms behind the impairment of the collagen-induced platelet  $\alpha$ -granule secretion that we found solely in platelets from the MA group remain unclear. One possibility is that it may involve an anomaly of the protein kinase C pathway associated with collagen-induced secretion.<sup>12</sup> It is not inconceivable that anomalies at different steps of the transformation of the cellular signal may have a different impact on the secretion from the two granule types, as the secretion from the dense and the  $\alpha$ -granules has been shown to differ with regard to dependence of  $Ca^{2+}$  and protein kinase C.<sup>10</sup> It seems less likely that this impairment of  $\alpha$ -granule secretion is due to a decreased amount of  $\alpha$ -granular material, because it was specific for collagen. Furthermore, it has been shown that the number of  $\alpha$ -granules is not decreased in patients with migraine.<sup>13</sup>

We failed to find any significant parallel changes in platelet aggregation induced by collagen or PAF. However, as quantitative measurement of platelet secretion is more sensitive than determination of platelet aggregation, it is possible that we may have failed to detect a parallel increase in platelet aggregation. Other studies have emphasised abnormalities in platelet aggregation in response to

PAF in patients with migraine,<sup>14,15</sup> and the issue needs further investigation.

The present findings of a specific platelet hypersensitivity to PAF in migraine, focuses attention on PAF as a possible contributor to platelet activation in migraine. To our knowledge, PAF concentrations have not been measured in migraineurs, but it is conceivable that migraine attacks may be associated with locally increased concentrations of PAF. There is evidence suggesting that the formation of PAF is stimulated by substance P<sup>16</sup>; a peptide that is released from the trigeminovascular system into the extracerebral circulation during migraine attacks.<sup>17</sup> In this context platelets are particularly implicated as target cells for PAF, because platelets are highly sensitive to PAF compared with other blood cells.<sup>18</sup> By inducing activation of normal human platelets in concentrations as low as 1–10 nM, PAF is the most potent platelet agonist known.<sup>6</sup> Release of  $\alpha$ -granular material from hypersensitive platelets may further enhance PAF formation, as  $\alpha$ -granule proteins may stimulate and act chemotactically on various inflammatory cells.<sup>19</sup> PAF is indeed a potent mediator in the CNS,<sup>20</sup> and further studies performed during attack may clarify the possible role of PAF in migraine.

The increased serotonin secretion found in platelets from patients with MA, was associated with similarly increased basal intraplatelet serotonin concentration as also indicated by our previous data.<sup>13</sup> Hence, the increased platelet serotonin secretion in MA seems to be due to increased platelet serotonin content rather than a hypersecretory response itself.

Our present results confirm previous findings that in MA there is an increased number of platelet dense granules, which contain the secretory pool of platelet serotonin.<sup>13</sup> This is not likely to be the sole explanation of the increased intraplatelet levels in MA, however, because the number of platelet dense granules was similarly increased in MwA. It is possible that the different platelet serotonin concentrations in MA and MwA may reflect different serotonin turnover in the two syndromes. Platelets have a rapid uptake mechanism for serotonin, and it is possible that increased platelet serotonin concentrations may be a reflection of increased circulating serotonin concentrations. This assumption is in agreement with the findings of Fontes Ribeiro *et al.*<sup>21</sup> of increased serum serotonin during the attack-free period in MA but not in MwA. Ferrari *et al.*<sup>22</sup> on the other hand, have reported that between attacks plasma serotonin concentrations were decreased in both types of migraine. In parallel, they found increased concentrations of the serotonin metabolite, 5-hydroxyindole acetic acid (5-HIAA), only in classic migraine, and it is possible that the discrepant findings may in part be because blood samples were taken at different time points, thus reflecting different phases of serotonin metabolism. By contrast with our present results Ferrari *et al.* also found normal platelet serotonin concentra-

tions in both migraine types. A factor that may in part explain why our findings differ is that we took into account variations in platelet serotonin during the menstrual cycle.

The diverse patterns of platelet content and secretion of serotonin in the two migraine types may be a reflection of different serotonin turnover, possibly also in the CNS. An increased serotonin turnover in MA may be related to the lower frequency, duration, and pain intensity of attacks reported in this migraine type<sup>23</sup>; also demonstrated in our patients (table 1). Further, considering that serotonin infusions have been shown to relieve migraine<sup>24</sup> an enhanced serotonin release from platelets may alleviate the MA attack.

Dr D'Andrea is a member of "Centro Interuniversitario di Ricerca Cefalee e Disordini Adattativi (UCADH)" (Interuniversity Center of Research in Headache and Adaptive Disorders).

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